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Effects of polyacrylamide and organic matter on microbes associated to soil aggregation of Norfolk loamy sand

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ABSTRACT

Polyacrylamide (PAM) has been reported to increase aggregation and improve soil physical properties in loamy sand soils, but nothing is known about the effects of PAM on microbes involved in aggregate formation. We studied the effects of PAM (0, 30 and 120 mg kg⁻¹) and organic matter (wheat [*Triticum aestivum*] and pecan [*Carya illinoensis*]) incubated in a Norfolk soil (a blend of 90% E horizon and 10% Ap horizon) for 96 days at 10% water content on microorganisms that have the ability to aggregate soil. We used an *in vitro* soil sedimentation assay to test the aggregative ability of the predominant, heterotrophic bacteria from the culturable portion of microaggregates (0.25–0.05 mm), fatty acid methyl ester (FAME) profiling to identify the isolates, and DNA sequencing to find their position in a phylogeny with known taxa. Among all the identified bacterial species, 19 were soil aggregators. There was a PAM effect across all residue types indicating that the highest proportion of soil aggregating isolates was at 120 mg kg⁻¹ PAM. This suggests that soil conditions created by the addition of 120 mg kg⁻¹ PAM favored the growth of bacteria functioning as soil aggregators. There was evidence of interaction between PAM and residue type. Differences were found in the PAM effect but only when no residue was added. The amount of soil aggregating basidiomycete fungi from the different aggregate size fractions was also determined using an enzyme-linked immunosorbent assay (ELISA). There was a higher amount of soil aggregating basidiomycete fungi in macroaggregates (2.00–1.00 and 1.00–0.50 mm classes) generated from PAM and/or wheat residue amended soil compared to the control soil, but no difference was found in treatments with pecan residue added. This suggests that PAM and PAM with wheat can be utilized as a source of nutrient for the basidiomycetes, but pecan appeared to inhibit their growth. This study is the first to provide evidence that adding PAM to soil favored the growth and survival of specific fungi and bacterial species functioning as soil aggregators *in vitro*.

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1. Introduction

Non-inversion deep tillage is a management practice commonly used in many agriculturally productive south-eastern Coastal Plain soils to disrupt the cemented subsurface of the E soil horizon which retards root growth and

decreases yield (Raper et al., 2000; Busscher et al., 2002). Amending soil with polyacrylamide (PAM), the flocculent polymer used in irrigation water to control soil erosion (Lentz and Sojka, 1994; Sojka et al., 1998), and/or organic matter (OM) has been proposed to replace this high cost tillage practice. Polyacrylamide was recently shown to

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improve soil physical properties (Busscher et al., 2006) and soil aggregation (Busscher et al., 2007) in sandy coastal soil and aggregate stability in various soil types (Green et al., 2004); thus using PAM could loosen the massive structure of the E horizon by reducing soil strength.

To develop methods to achieve and maintain a long-lasting satisfactory degree of aggregate stability, it is important to understand the effects of PAM and/or OM on the populations of microbes that have the potential to function as soil aggregators, and whether PAM and OM can be used as nutrient sources for the growth and survival of these microorganisms. Our hypothesis was that the addition of PAM and/or OM to Norfolk loam sandy soil would increase the proportion of bacteria and fungi that have the potential to aggregate and stabilize soil.

The high structural stability of microaggregates (0.25–0.05 mm) has been attributed to the presence of bacteria cells embedded in capsules of extracellular polymeric substances (EPS) often surrounded by a layer of clay particles (Foster, 1981). To gain insight into possible mechanisms by which soil particles are stabilized within microaggregates, we sought to examine the species composition of bacteria and determine whether these bacteria were capable of binding soil particles to elucidate the key microbial effectors of soil aggregation for potential large-scale growth in future soil testing and application. In this study, cultural methods were used to procure adequate biomass of bacteria occurring at the highest population levels in microaggregates of PAM and/or OM treated soil and an *in vitro* assay was developed to screen for potential soil aggregating species.

While endomycorrhizal fungi have been described as the apparent driving force in soil aggregation (Wright and Upadhyaya, 1996), reports have demonstrated that basidiomycete fungi can produce EPS with soil binding activities (Caesar-Ton-That, 2002) and play an important role as soil aggregators (Tisdall et al., 1997; Caesar-Ton-That and Cochran, 2000). Furthermore, many basidiomycetes inhabiting fragmentary soil litter are responsible for decomposition and nutrient-release processes and are likely to be the dominant recyclers of plant residue (Boosalis et al., 1967; Ploetz et al., 1985) because they can secrete extracellular ligninolytic enzymes (laccase and peroxidase) for the degradation of complex molecules (Hammel, 1995; Hibbet and Thorn, 2001; Thorn et al., 1996). Methods of detection and quantification of the amount of soil aggregating basidiomycete fungi in the aggregate fractions of soil treated with PAM and/or OM would provide useful information on whether these specific fungi can grow and utilize these soil amendments as a source of nutrients.

The objectives of this study were to (1) identify the predominant heterotrophic and aerobic bacteria from the microaggregates (0.25–0.05 mm) of Norfolk loamy sand treated with 3 rates of PAM (0, 30 and 120 mg kg⁻¹) and/or 2 types of organic residue (wheat [*Triticum aestivum*] and pecan [*Carya illinoensis*]), (2) develop a sedimentation assay to measure the soil aggregative function of the bacterial isolates, and (3) detect and quantify the soil aggregating basidiomycete fungi in 3 macroaggregates size classes (2.00–1.00, 1.00–0.50, and 0.50–0.25 mm) generated from the different treatments.

2. Materials and methods

2.1. Soil collection

Soil used in the experiment was Norfolk loamy sand (fine-loamy, siliceous, thermic Typic Kandiodult in the USDA classification or an Acrisol in the FAO classification) formed in Coastal Plain marine sediments. Over the years, the Ap horizon had been tilled to a depth of 0.20 m. Below the plow layer, the soil had an eluviated E horizon that restricted root growth and typically extended to depths of 0.30–0.45 m. General characteristics for Ap and E horizons were similar with differences based mainly on surface organic matter. The Ap and E horizons had 1–3 cmol kg⁻¹ cation exchange capacity, 20–80 g kg⁻¹ clay, and 2–20 g kg⁻¹ of organic matter (Soil Survey Staff, 2006). To remove non-soil materials and break up the soil, both horizons were air dried and pushed through a 2 mm sieve. In this experiment, the Norfolk E horizon was mixed into a blend of 90% E horizon and 10% Ap horizon material according to Busscher et al. (2006). The 10% Ap horizon was added to assure that the soil would have a microbial presence that could decompose OM. The soil mixture had 66% sand, 30% silt, and 3.8% clay. The OM content was 0.19% for the mixture.

2.2. Incubation study

The incubation study had a completely randomized factorial design with five organic matter treatments and three PAM levels. The five organic matter treatments were a (1) control, ground wheat (*T. aestivum*) stubble applied at (2) 3.22 (w1) or (3) 6.44 g kg⁻¹ (w2) and ground pecan (*C. illinoensis*) applied at (4) 3.52 (p1) or (5) 7.04 g kg⁻¹ (p2). The three PAM treatments were 0 (0 PAM), 30 (30 PAM), or 120 mg kg⁻¹ (120 PAM) which had C:N ratios adjusted to 20:1 by adding NH₄NO₃ in amounts of 0.157, 0.307, 0.456, 0.318, and 0.476 g kg⁻¹, respectively. Four hundred and fifty grams of treated or untreated soil were packed into 100 mm diameter pots with a 20 mesh nylon screen on the bottom to prevent soil loss from drain holes. The soil was approximately 10 cm deep after packing. Soil was packed to a bulk density of approximately 1.2 Mg m⁻³. The PAM formulation was an anionic formulation of molecular size 12 MDa and 35% charge density (SNF Inc, Riceboro, GA, USA). Because a small amount of PAM was added to the soil, it would not mix uniformly in a dry state. For each treatment, an appropriately diluted solution was sprayed onto soil that had been spread out on a table; soil and solution were gently mixed on waxed paper.

Treatments were incubated in a laboratory for 96 days at 20–22 °C and ambient humidity. Treatments were maintained at 10% soil water content on a dry weight basis by weighing and adding deionized water to the pots 2–3 times a week. Each treatment was replicated three times. At the end of the experiment, dry soil (100 g) from each treatment was placed into a nest of sieves with openings 2.00, 1.00, 0.50, 0.25, and 0.05 mm to generate aggregate sizes classes using the procedure of Sainju et al. (2003).

2.3. Isolation of bacteria

Isolation of the predominant bacteria in the microaggregates was performed according to Caesar-TonThat et al. (2007).

Briefly, 1 g of microaggregates (0.25–0.05 mm size class) was agitated in MgSO_4 buffer (0.1 M, pH 7.3) with glass beads (0.5 mm) for 6 h at 4 °C on a shaker at 200 rpm. Suspensions were diluted by a factor of 100 with buffer and 150 μL aliquots of soil suspensions were plated on 0.3% Tryptic Soy Broth Agar (TSBA) using a spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK). Thus, 10^5 cells g^{-1} soil represented a typical population level at which the colonies which were considered the predominant isolates were obtained. Four plates were made for each replicated sample, for a total of 12 plates for each treatment. Plates were incubated for 24–48 h at 28 °C. Five colonies at the end of the spiral from each plate were collected, for a total of 60 colonies per treatment. A total of 900 colonies were isolated from the 15 treatments, purified then stored at –80 °C in Luria-Bertani medium amended with 15% glycerol. All the isolates were analyzed for their FAME profiles.

2.4. Identification by FAME and DNA analyses

FAME profiles were routinely used to identify bacteria (Cavigelli et al., 1995; Ibekwe and Kennedy, 1999). FAMES were obtained by saponification, methylation and extraction following the MIDI system (Microbial Identification System, Inc., Newark, NJ, USA). MIDI Microbial Identification Software (Sherlock Aerobic Bacterial TSBA50 Library) was used for the identification of the isolates. *Bacillus maroccanus* (ATCC#25099) and *Stenotrophomonas maltophilia* (ATCC#13637) were used as references. Only strains with the similarity index (SIM) ≥ 0.500 were considered a good match (Oka et al., 2000). Isolates that had SIM < 0.500 or could not be identified by FAME due to lack of information in MIDI Library TSBA50 were not investigated.

To corroborate identifications made by MIDI, molecular-based methods of identification were additionally used. Isolate DNA was extracted using a Qiagen (Valencia, CA, USA) DNeasy Tissue kit. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene region used primers 16S-27f (5'-GAGTTTGATCCTGGCTCAG-3') and 16S-960r (5'-GCTTGTGCGGGYCCCCG-3') with the following cycling conditions: 95 °C (10 min); 25 cycles of 94 °C (30 s), 56 °C (30 s), 72 °C (2 min); and then 72 °C (2 min). A 50 μL reaction was performed for each isolate, and PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA). Purified templates were sequenced in two directions with either a CEQ 2000XL (Beckman Coulter Inc., Fullerton, CA, USA) or an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA), using the same primers listed above. Isolate DNA sequences generated in this study are available from the authors, and were aligned using CLUSTALW (Thompson et al., 1994). Maximum Parsimony (MP) analysis of the data set was performed using PAUP v. 4.0b8 (Swofford, 2000). The heuristic MP search employed 500 random taxon addition sequences and the tree-bisection-reconnection (TBR) branch-swapping algorithm. All characters were weighted equally and insertion/deletion events, no matter what their length, were treated as one mutational event, as in Simmons and Ochoterena (2000). A 10,000 replicate “fast” stepwise-addition bootstrap analysis was conducted to assess clade support.

2.5. Sedimentation assay

All the isolates belonging to identified species from each of the 15 treatments were assayed to determine their potential to aggregate soil in solution. In the case of *Bacillus cereus*, *Bacillus megaterium*, *Kurthia sibirica*, and *Paenibacillus alvei*, only 2 identified isolates with the highest SIM of each of the treatments were assayed due to their high number. A total of 349 isolates were assayed. To avoid soil aggregation due to the cohesive properties of the clay and the capability of clay in loam soils to shrink and swell (Denef et al., 2002), bulk soil (14% clay, 14% silt, and 72% sand) obtained along a stream bank at Sidney, MT, USA was used for the assay. The deposited soil is fluvial in origin; therefore, no soil series name is available. Soil was sieved to obtain <0.05 mm aggregate size class. Bacterial cells from 48 to 72 h-old cultures grown on 0.3% TSBA were weighed (100 mg) and washed once with deionized water to eliminate any residual nutrients from the culture medium that could result in different qualitative carbon inputs which could introduce artifacts in the binding process to soil particles. In previous studies which included a washing step with buffer (Li and Logan, 2004) or distilled water (Jucker et al., 1996), it has been shown that such washes did not affect the attachment of EPS producing bacteria to many surfaces. Cells were counted using a hemacytometer before adding to borosilicate glass tubes (20 mm \times 150 mm) containing 10 mL of deionized water and 1.25 g of <0.05 mm fraction sieved soil to obtain final cell concentrations of either 10^6 , 10^5 , or 10^4 cells mL^{-1} . These cell concentrations were chosen because they represented minimum cell concentrations at which sedimentation of soil particles could occur, as established in preliminary studies with a range of bacteria species. The sedimentation assay was repeated 3 times for each cell concentration. A set of controls was prepared in a similar manner but without the addition of bacteria.

Tubes containing the mixture of soil particles and bacteria were vortexed for 10 s at 2250 rpm and the mixture was allowed to settle for 5 and 10 min at room temperature. Images of the reflected light (Universal/Hi-vision fluorescent light F32T8/TL735, Philips, NY, USA, light intensity 7.435 ± 0.064 Rad [watt m^{-2}]) were captured using a digital camera (Nikon, model D-80) with night vision settings (near infrared, 800–1000 nm) at 24.78 ± 0.746 °C. The captured image was calibrated by referring white (255 in gray-scale value) and black (0 in gray-scale value) image spots as 100% and 0% reflectance, respectively. Adobe Photoshop software (version 7.0) was used for the conversion of the images to a gray-scale value for each target solution into the reflectance (expressed in %) that directly correlates to relative differences of the solution density. Means reflectance measurements from isolates of the same species were compared to the other species and to a control without bacteria added.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The ELISA protocol used to detect and quantify soil aggregating basidiomycete fungi in aggregate size fractions was described in details by Caesar-TonThat et al. (2001). Briefly, aggregates (2.00–1.00, 1.00–0.50, and 0.50–0.25 mm classes) were homogenized in carbonate buffer (20 mM NaHCO_3 ,

28 mM Na₂CO₃, pH 9.6). The supernatant was serially diluted then replicated samples of each of the dilutions were added to a 96-well flat-bottom microtiter plate (Immulon IV, Thermo LabSystems, Boston, MA, USA) and incubated for 16 h. The plates were washed with phosphate buffered saline-Tween-20 (PBST, pH 7.4) then incubated with a non-specific binding blocking agent (Teleostean gelatin, 1% in PBST). Polyclonal antibodies raised against soil aggregating basidiomycete cell walls were used as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit polyspecific immunoglobulins (Sigma, St. Louis, MO) as secondary antibodies. A solution of 3,3',5,5' tetramethylbenzidine and 0.02% H₂O₂ (ImmunoPure TMB Substrate Kit, Pierce, Rockford, IL, USA) was used as substrate and H₂SO₄ (2.5 M) to stop the reaction. Absorbance was read at dual wavelengths of 450/655 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The amount of fungi was expressed in $\mu\text{g g}^{-1}$ soil based on a standard curve generated from known amounts of fungal antigens. The aggregate samples were analyzed 3 times.

2.7. Statistical analysis

Honesty significant difference procedure of Tukey–Kramer, standard ANOVA was performed on soil sedimentation data with statistical differences evaluated at $P < 0.01$ and on fungal quantification data at $P < 0.05$ using the JMP statistical software package (version 6.0, 2005, SAS Institute Inc., Cary, NC, USA). PAM and residue type were analyzed as factors affecting the proportion of bacterial isolates that can function as soil aggregators using a generalized linear model. PROC GLIMMIX (SAS Institute Inc., Cary, NC, USA) was used to fit proportion of aggregating isolates on PAM, residue type and interaction of PAM and residue type using a logit model (Littell et al., 2006).

3. Results

The *in vitro* sedimentation assay performed on 349 isolates of the 43 identified species that dominated the microaggregates from all the treatments (Table 1) indicated that 19 (18 Gram-positive [GP] and 1 Gram-negative [GN]) soil aggregating species can be differentiated from 24 non-aggregating species. For example, assays on identified isolates belonging to *Paenibacillus alvei* showed no significant difference in reflectance measurements with the control (50.53% vs. 46.43%, respectively, using 10^6 cells mL⁻¹ and 5 min of soil settlement) whereas, assays with *Bacillus cereus* isolates indicated significantly higher reflectance than the control (97.60% vs. 46.43%, respectively, using 10^6 cells mL⁻¹ and 5 min of soil settlement). Among the isolated GN species, only *Pseudomonas huttiensis* could aggregate soil.

From the total 900 isolates of the 15 treatments, only 829 isolates could be purified and identified by FAME (Table 2). In all the treatments, the majority of the identified species were GP. FAME identified 40 GP species from 9 genera, *Arthrobacter* (2.41% of the total isolates), *Bacillus* (78.53%), *Brevibacillus* (0.60%), *Kocuria* (0.24%), *Kurthia* (5.91%), *Microbacterium* (3.14%), *Micrococcus* (0.72%), *Paenibacillus* (7.48%), and *Rothia* (0.36%) and 3 GN species from genera *Shewanella* (0.12%), *Paucimonas*

(0.24%), and *Pseudomonas* (0.48%). Table 2 indicates the number of the predominant isolates per species identified by FAME from all the 3 replications combined of each treatment and their relation to soil aggregative ability. In the control treatment with no amendments, *Bacillus cereus* and *Paenibacillus pabuli* were the only soil aggregators identified (species demonstrated to have soil aggregating potential are shown in shading in Table 2) whereas, in all the other treatments there were more types of soil aggregating species; for example, in treatments with 120 mg kg⁻¹ PAM added (120, none), 6 different soil aggregating species (*Bacillus* GC group 22, *Bacillus borstelensis*, *Bacillus cereus*, *Bacillus pumilus*, *K. sibirica*, and *Paenibacillus polymyxa*) were identified.

To corroborate the identification by FAME of the soil aggregating species, the 16S rRNA amplification region of 18 GP species with highest SIM identified by FAME (ID number of species are indicated in bold in Table 2) were sequenced and analyzed. Fig. 1 shows the position of the isolates in the maximum parsimony tree showing the relationship to reference GP bacteria. In general, DNA analysis of the soil aggregating isolate sequences matched with the FAME analysis. Among the few discrepancies found between the 2 approaches of identification, DNA analysis placed the SC13-8931 isolate (*Voriovorax erythromyxa* with SIM 0.638 by FAME) in the *Bacillus* group and the SC5-9001 isolate (*Paenibacillus pabuli* with SIM 0.635 by FAME) in the *Brevibacillus* group.

The results of the model fit on the proportion of soil aggregating isolates on PAM, residue type and interaction of PAM and residue type are provided in Table 3. There was evidence of interaction between PAM and residue type at $P < 0.0904$. Differences were found in PAM effect but only for no residue added. The PAM effect across all residue type indicated that the highest proportion of soil aggregating isolates was with 120 mg kg⁻¹ PAM.

Fig. 2 indicates the distribution of the soil aggregating basidiomycete fungi in the aggregates size fractions from the various treatments detected by ELISA. There was an increase of 1.75-fold compared to the amount in control non-amended soil. A significant elevated amount of antigens was detected in macroaggregates of size classes 2.00–1.00 and 1.00–0.50 mm of PAM-treated soil. Addition of wheat to 120 PAM-treated soil also provoked a significant increase of antigens in the large aggregate size class of 2.00–1.00 mm but the increase was less compared to soil treated with only PAM. There was no antigenic response when pecan was added to PAM-treated soil.

4. Discussion

Various physical measurement techniques including resistance to pressure of artificial soil aggregates (Caesar-TonThat et al., 2007) or biofilms (Chen et al., 1998; Ahimou et al., 2007) amended with individual bacterial species have been used to elucidate the degree of adhesiveness of the binding agents produced by the microorganisms. In this study, an *in vitro* assay consisting of mixing fine soil particles with individual isolates of bacteria from pure cultures in water and measuring the soil density of the mixture by reflectance, has allowed the predominant heterotrophic and culturable bacteria isolated

Table 1 – Soil sedimentation assay of the predominant species isolated from PAM and/or OM treated soil and untreated soil, and identified by fatty acid methyl ester (FAME) profiling

Species	% Reflectance					
	Bacterial concentration (cells/mL)					
	10 ⁶	10 ⁵	10 ⁴	10 ⁶	10 ⁵	10 ⁴
		5 min			10 min	
Gram positive						
<i>Arthrobacter globiformis</i> (2) [§]	40.22	43.33	44.09	42.66	51.87	73.28
<i>Arthrobacter mysorens</i> (2)	40.99	37.79	42.54	43.50	49.31	55.84
<i>Arthrobacter ramosus</i> (2)	48.43	40.58	42.06	56.43	53.30	58.49
<i>Arthrobacter viscosus</i> (14)	39.25	56.10	54.96	38.58	60.29	71.63
<i>Bacillus</i> GC group 22 (18)	*91.11	*63.83	38.57	*94.73	72.83	52.55
<i>Bacillus alcalophilus</i> (6)	*92.16	*68.06	54.90	*89.03	74.00	67.04
<i>Bacillus amyloliquefaciens</i> (2)	54.74	43.48	47.30	70.18	64.96	73.54
<i>Bacillus atrophaeus</i> (5)	56.68	56.46	55.32	78.68	68.52	67.84
<i>Bacillus azotoformans</i> (4)	*97.20	*71.48	*59.29	*95.88	79.71	72.59
<i>Bacillus borstelensis</i> (32)	*94.13	*62.31	37.76	*94.88	69.30	47.18
<i>Bacillus centrosporus</i> (1)	56.62	54.06	52.65	81.11	72.08	72.11
<i>Bacillus-cereus</i> (28)	*97.60	*84.36	*63.20	*95.92	*90.28	*84.00
<i>Bacillus flexus</i> (8)	55.24	41.72	33.58	59.81	54.48	47.15
<i>Bacillus formosus</i> (2)	*96.89	*81.81	50.53	*97.16	*86.09	61.95
<i>Bacillus globisporus</i> (2)	57.67	53.98	45.37	58.82	59.54	56.45
<i>Bacillus gordonae</i> (8)	*97.42	*69.94	*66.71	*96.63	77.79	75.45
<i>Bacillus lacterosporus</i> (2)	*97.57	*75.51	*64.58	*95.66	77.66	71.90
<i>Bacillus macerans</i> (16)	*93.81	*66.69	32.73	*94.86	79.08	49.04
<i>Bacillus megaterium</i> (30)	54.31	50.58	46.83	69.82	64.01	66.85
<i>Bacillus niacini</i> (4)	52.95	39.48	39.46	57.37	48.77	55.52
<i>Bacillus oleronius</i> (1)	53.74	49.61	47.75	65.55	59.16	65.60
<i>Bacillus parabrevis</i> (4)	*93.28	*71.49	*64.27	*93.62	*84.77	79.11
<i>Bacillus pumilus</i> (29)	*81.12	*63.02	*59.27	*84.10	73.00	76.22
<i>Bacillus sphaericus</i> GC subgroup C (1)	54.83	52.19	53.21	63.11	66.15	70.56
<i>Bacillus sphaericus</i> GC subgroup F (7)	57.31	56.65	52.86	75.58	74.06	77.40
<i>Brevibacillus choshinensis</i> (1)	57.14	54.93	53.66	75.23	70.01	78.64
<i>Brevibacillus liquefaciens</i> (1)	53.71	48.94	49.11	70.04	63.06	72.43
<i>Brevibacillus reuszeri</i> (3)	55.99	57.15	49.35	68.66	67.76	68.76
<i>Kocuria erythromyxa</i> (2)	*93.66	*60.52	44.94	*90.80	64.52	58.97
<i>Kurthia gibsonii</i> (10)	*83.85	*59.78	45.46	*90.20	69.62	56.62
<i>Kurthia sibirica</i> (26)	*91.79	*65.30	53.83	*94.23	71.18	69.61
<i>Microbacterium barkeri</i> (8)	53.01	42.63	34.46	69.14	56.47	59.12
<i>Microbacterium laevaniformans</i> (18)	35.79	31.82	47.60	37.18	35.99	52.95
<i>Micrococcus-luteus</i> (4)	55.46	42.92	44.13	60.22	53.26	63.14
<i>Paenibacillus-alvei</i> (22)	50.53	45.14	50.48	53.53	52.69	60.44
<i>Paenibacillus chondroitinus</i> (2)	*73.95	*62.31	54.37	*81.85	66.82	73.61
<i>Paenibacillus maquariensis</i> (1)	52.64	37.91	46.87	59.29	48.04	66.25
<i>Paenibacillus pabuli</i> (4)	*69.78	*74.22	*63.29	*81.92	80.51	74.87
<i>Paenibacillus polymyxa</i> (6)	*76.55	*69.95	*61.59	*83.78	80.68	80.34
<i>Rothia dentocariosa</i> (4)	*96.55	*63.46	52.16	*96.89	73.53	68.11
Gram negative						
<i>Shewanella putrefaciens</i> (1)	55.43	55.31	55.27	78.14	71.24	76.85
<i>Paucimonas lemoignei</i> (2)	57.88	48.38	37.10	65.09	62.20	54.94
<i>Pseudomonas huttiensis</i> (4)	*93.34	*86.45	*66.67	*94.05	*90.82	76.41
Control	46.43	46.43	46.43	68.61	68.61	68.61

[§]The number of identified isolates assayed for soil sedimentation is indicated between parentheses. Within a column of each dilution and sedimentation time, numbers followed by an asterisk (*) are significantly higher than the control (not exposed to bacteria) at P < 0.01. Species indicated in shading are considered soil aggregators.

from microaggregates to be characterized for their capability to bind soil.

Compared to the widely used technique of light transmittance to estimate the density of solution or aggregates

(Dontsova and Norton, 2002; Joyce and Smith, 2003), the technique of light reflectance used in this study offered the advantage of being quick and cost-effective because no special expensive equipment or time-consuming dilution procedures

Table 2 – Number of isolates per species identified by fatty acid methyl ester (FAME) profiling from microaggregates of PAM and/ or OM-amended soil and non-amended soil, and their relation to soil aggregative ability

Isolates identified by FAME	Treatments															Isolates used for DNA analysis
	0, none	30, none	120, none	0, w1	30, w1	120, w1	0, w2	30, w2	120, w2	0, p1	30, p1	120, p1	0, p2	30, p2	120, p2	
GP																
Arthrobacter globiformis														2		
Arthrobacter mysorens		2														
Arthrobacter ramosus					2											
Arthrobacter viscosus	2	2		2	2					6						
Bacillus GC group 22			2	2	2				4	2			6			
Bacillus alcalophilus						2						2	2			
Bacillus amyloliquefaciens											2					
Bacillus atrophaeus						4								1		
Bacillus azotoformans										2			2			
Bacillus borstelensis		4	4	4	6	2	2	2		2		4		2		
Bacillus centroporus	1															
Bacillus cereus	6	8	16	12	4	5	12	6	4		6	2	2	8	8	
Bacillus flexus	2			2	4											
Bacillus formosus						2										
Bacillus globisporus	2															
Bacillus gordonae											2	2	2		2	
Bacillus lacterosporus										2						
Bacillus macerans		2						2					3	5	4	
Bacillus megaterium	24	21	19	22	23	23	33	28	29	30	28	29	34	27	30	
Bacillus niacini					4											
Bacillus oleronius									1							
Bacillus parabrevis						2				2						
Bacillus pumilus			2				2		8	2	7	2		4	2	
Bacillus sphaericus GC subgroup C							1									
Bacillus sphaericus GC subgroup F				2		4				1						
Brevibacillus choshinensis				1												
Brevibacillus liquefaciens		1														
Brevibacillus reuszeri						1	2									
Kocuria erythromyxa					2											
Kurthia gibsonii						2					5				3	
Kurthia sibirica		4	2		2	4	2	4	4	4	2	4	3	3	1	
Microbacterium barkeri					1			4	3							
Microbacterium laevaniformans			1	2	2			4			2	7				
Micrococcus luteus																4
Paenibacillus alvei	12	8	4	6	1	4	2		4	2			4		2	
Paenibacillus chondroitinus								2								
Paenibacillus macquariensis								1								
Paenibacillus pabuli	2			2												
Paenibacillus polymyxa		2	2			2										
Rothia dentocariosa		2		1												
GN																
Shewanella putrefaciens							1									
Paucimonas lemoignei	2															
Pseudomonas huttiensis								4								*
Total of isolates analyzed	53**	56	52	58	55	57	57	57	57	55	54	52	58	52	56	

*Organic matter added: 0 g kg⁻¹ (none), 3.22 g kg⁻¹ (w1) and 6.44 g kg⁻¹ (w2) ground wheat stubble, 3.52 g kg⁻¹ (p1) and 7.04 g kg⁻¹ (p2) ground pecan. PAM added: 0 mg kg⁻¹ (0), 30 mg kg⁻¹ (30), and 120 mg kg⁻¹ (120).

^a Isolates with similarity index (SIM) > 0.500 were considered a good match (Oka et al., 2000) and were considered in the analysis. Species indicated in shading were determined soil aggregators (Table 1).

^c Isolates belonging to each of the soil aggregating species identified by FAME with the highest SIM were DNA sequenced and analyzed (Fig. 1). Number between parentheses indicates the highest SIM.

* Data of DNA analysis not presented.

**Number of isolates pooled over 3 replications.

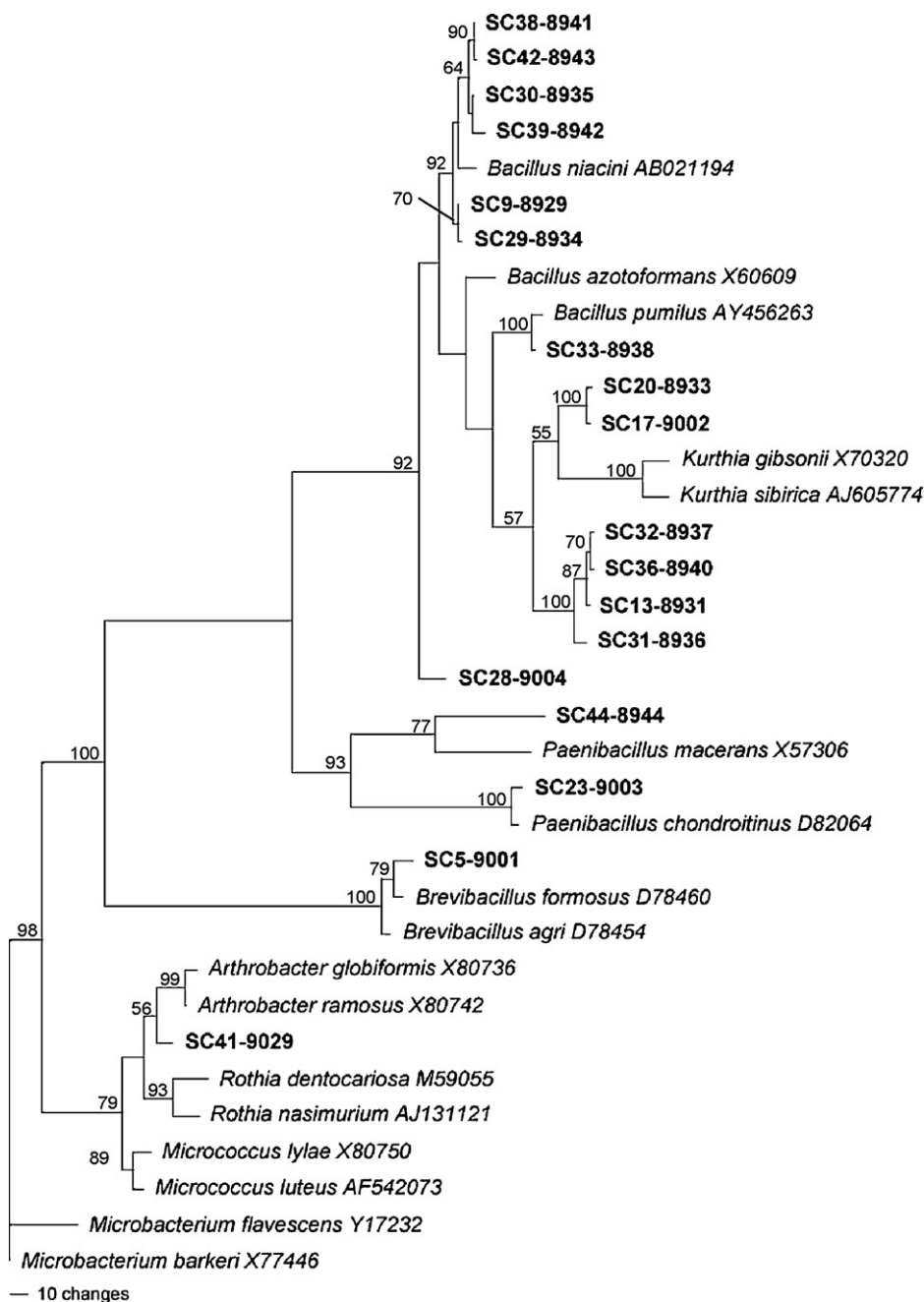


Fig. 1 – Single most parsimonious tree, 1013 steps in length, resulting from the analysis of 16 S rRNA gene sequences (852 aligned bases; 317 of these parsimony informative) from 35 Gram-positive soil-aggregating bacteria. Isolates from this study are shown in bold, while taxa used as phylogenetic placeholders (shown in italics, with GenBank accession numbers following specific epithet) are from the Ribosomal Database Project II Hierarchy Browser collection of sequenced Type Strains (Cole et al., 2003). Bootstrap values (>50%) are shown above branches.

were required. Also, the technique of transmittance is often limited by a short measurement range (Gributs and Burns, 2004). Measurement of light reflectance has been used in association with remote sensing technology, for example, to analyze reflected solar radiation from crop canopies to search for insect outbreaks (Riedell and Blackmer, 1999).

The significantly higher proportion of soil aggregating isolates from microaggregates of Norfolk loamy sand amended with 120 mg kg⁻¹ PAM for 96 days than in una-

mended soil suggests that adding PAM to soil can provide adequate environmental and nutritional conditions that favored bacteria with soil aggregative potential to grow and dominate in the microaggregates. However, there were differences in the PAM effect on the proportion of soil aggregating isolates only when no OM (wheat or pecan) was added, indicating that OM might interfere with the effect of PAM. It has been reported that, when incorporated into soil, PAM experienced degradation rates of 10% per year as a result

Table 3 – Effect of PAM and organic residue^a on the proportion of the predominant soil aggregating bacteria isolated from microaggregates of Norfolk loamy sand

Test of fixed effects	Number	DF	F value	Pr > F
PAM	2	30	3.86	0.0323
Residue type	4	30	0.28	0.8884
PAM Residue type	8	30	1.94	0.0904
Test of simple effects of PAM for each residue type				
None	2	30	7.89	0.0018
w1	2	30	1.48	0.2442
w2	2	30	0.41	0.6701
p1	2	30	0.46	0.6371
p2	2	30	0.1	0.9013
Comparison of PAM for no residue			Proportion	
0, none			0.1509 b ^{**}	
30, none			0.3929 ba	
120, none			0.5385 a	

^a PAM added: 0 mg kg⁻¹ (0), 30 mg kg⁻¹ (30), and 120 mg kg⁻¹ (120). Organic residue added: 0 g kg⁻¹ (none), 3.22 g kg⁻¹ (w1) and 6.44 g kg⁻¹ (w2) ground wheat stubble, 3.52 g kg⁻¹ (p1) and 7.04 g kg⁻¹ (p2) ground pecan.

^{**} Letter grouping was determined using Tukey–Kramer's honestly significant difference (HSD) procedure ($P < 0.05$) on parameter estimates from logit model.

of physical, chemical, biological and photochemical processes (Azzam et al., 1983), which is slower than OM. Furthermore, PAM is slow to break down because microbial and chemical attack is only on the end of the polymer (Kay-Shoemaker et al., 1998). Thus, it is reasonable to postulate that PAM may be still available after 96 days as a substrate to support the growth of the soil aggregating isolates, resulting in a higher proportion of these isolates than in soil without PAM added. However, adding OM to PAM-treated soil may stimulate a flush of microbial activity that can accelerate the decomposition of

PAM, thus resulting in nutrient depletion after 96 days. This could explain how the PAM effect on the proportion of soil aggregating bacteria depends on OM.

The elevated amount of soil aggregating basidiomycete fungi in the macroaggregate size classes of 2.00–1.00 and 1.00–0.50 mm of PAM-treated soil compared to the non-treated control soil suggests that PAM favored the growth and survival of these specific fungi by providing them the necessary substrate. Basidiomycetes are slow growing fungi known to produce enzymes that can catalyze not only the oxidation of a large number of phenolic but also non-phenolic complex substrates (reviewed by Bennet et al., 2002), however, nothing is known concerning the enzymatic biodegradation of PAM to acrylamide by these fungi. Since several authors demonstrated that basidiomycetes are able to cleave the aliphatic C–C bonds of polyethylene and polyethylene oxide molecules (Kerem et al., 1998; Iiyoshi et al., 1998), we speculate that these fungi could cleave the ethylene back bone of PAM polymer, and utilize PAM as a source of nutrient for their growth. In addition to the role of PAM in flocculating soil, these fungi present in the aggregates could also contribute to the increase in aggregation of soil treated with PAM (Busscher et al., 2007) since they can produce polysaccharides that act as soil binding agents (Caesar-Ton-That, 2002).

Antigenic response of the large and medium macroaggregates (2.00–1.00 and 1.00–0.50 mm classes) from 30 or 120 mg kg⁻¹ PAM-treated soil with wheat added was lower than 30 or 120 mg kg⁻¹ PAM-treated soil without wheat added. Addition of wheat residues possibly induced growth of fast-growing microorganisms that are more competitive than the basidiomycete fungi, resulting in a decrease in the amount of these specific fungi. ELISA indicated no antigenic response when pecan residues were added to PAM-treated soil. We found it interesting that combined pecan and PAM amendments of soil did not support increased populations of these specific organisms, and may even depress them slightly. Pecan

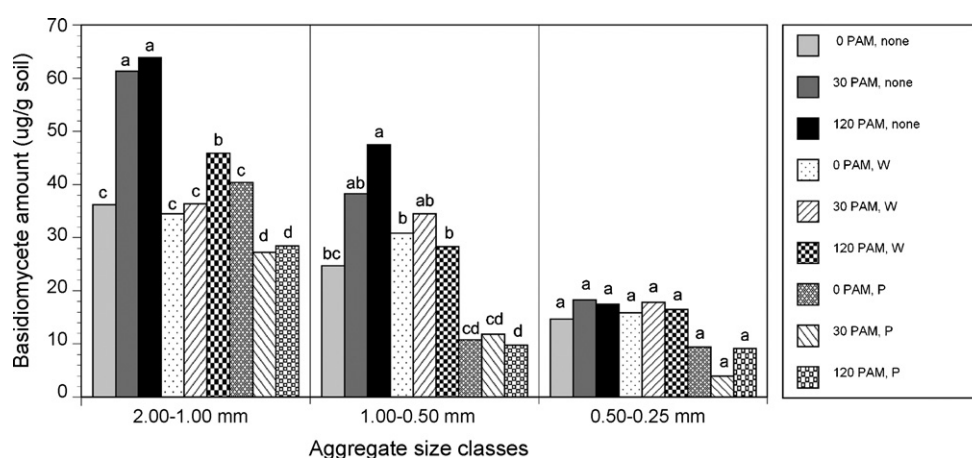


Fig. 2 – Relative distribution of soil aggregating basidiomycete antigens in aggregates recovered from three different size classes (2.00–1.00, 1.00–0.50, and 0.50–0.25 mm) of PAM and/or OM treated Norfolk loamy sand. Values are means ($n = 18$, except $n = 12$ for treatment 0 PAM). Organic matter added was of 0 g kg⁻¹ (none), 3.22 g kg⁻¹ (w1) and 6.44 g kg⁻¹ (w2) ground wheat stubble, 3.52 g kg⁻¹ (p1) and 7.04 g kg⁻¹ (p2) ground pecan. PAM added was 0 mg kg⁻¹ (0 PAM), 30 mg kg⁻¹ (30 PAM), and 120 mg kg⁻¹ (120 PAM). For each aggregate size class, numbers followed by different letters are significantly different using Tukey–Kramer (HSD) test with $P < 0.05$. For presentation, data of treatments w1 and w2, and p1 and p2 were combined.

trees are known to produce juglone, a naphthoquinone that has broad-spectrum antimicrobial activity (Brigham et al., 1999; Appleton et al., 2000). We speculate that this compound in PAM-treated soil caused growth inhibition of basidiomycetes in the aggregates.

In this study, the predominant, aerobic, and heterotrophic bacteria have been isolated from the cultural portion of soil microaggregates (0.25–0.05 mm) of Norfolk loamy sand amended with PAM and/or OM. Cultural approaches can be justified in this case because growth *in vitro* to provide adequate biomass and purity of the isolates are needed (Ritz, 2007; Nichols, 2007; Ellis et al., 2003) in order to apply the sedimentation assay on the individual isolates. Although our study is based on the subset of bacteria that are capable of growing on 0.3% TSBA at 28 °C in an aerobic environment, and probably the dominant number of species that have been isolated can be misrepresented, this medium has proven to be commonly used to isolate numerous bacterial species involved in key nutrient cycles (Heylen et al., 2006), species in contaminated soils (Ellis et al., 2003), or species involved in bioremediation (Daane et al., 2003). Future studies could attempt to match the *in vivo* and the *in vitro* conditions to better mimic conditions existing in the habitat from which the isolates were obtained to increase the likelihood of retrieving more species; for example, combining cultivation with simulation of natural growth conditions including use of natural media (Kaeberlein et al., 2002) or addition of growth substrates to culture medium (Davis et al., 2005).

Results of this study have provided the foundations for the next experimental approach which would be to reintroduce individual or a consortium of isolates of interest (as formulated inocula, pure cultures or other delivery methods) to the same type of soil under differing management to investigate the fate of these introduced species in correlation with soil aggregation.

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